ambiguities according to ref. 29. The null hypothesis of no geographical association of clades and nested clades was tested by permutation of clades against sampling locations for tip and interior clades in the program GeoDis ver. 2.0 (ref. 30). The biological interpretation of the results was done following the inference key of ref. 15.

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Evolution of cooperation and conflict in experimental bacterial populations

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A fundamental problem in biology is the evolutionary transition from single cells to multicellular life forms[1–3]. During this transition the unit of selection shifts from individual cells to groups of cooperating cells[1,3,4]. Although there is much theory[5–15], there are few empirical studies[16]. Here we describe an evolutionary transition that occurs in experimental populations of *Pseudomonas fluorescens* propagated in a spatially heterogeneous environment[17]. Cooperating groups are formed by over-production of an adhesive polymer[18], which causes the interests of individuals to align with those of the group. The costs and benefits of cooperation, plus evolutionary susceptibility to defecting genotypes, were analysed to determine conformation to theory[1,3,12]. Cooperation was costly to individuals, but beneficial to the group. Defecting genotypes evolved in populations founded by the cooperating type and were fitter in the presence of this type than in its absence. In the short term, defectors sabotaged the viability of the group; but these findings nevertheless show that transitions to higher orders of complexity are readily achievable, provide insights into the selective conditions, and facilitate experimental analysis of the evolution of individuality.

Multicellularity has evolved independently on several occasions and is likely to have simple, albeit diverse, explanations[1,2]. Until now, attention has focused on the advantages of multicellularity and its implications for the development of complexity[1,2,19–21]. Less consideration has been given to the selective conditions necessary for the evolutionary origin of simple undifferentiated groups: these have special significance because they may have been the raw material for the evolution of multicellular organisms[2,15,22].

The origin of cooperating groups of cells requires an understanding of how selection operates at the level of individual cells[1,3,6,8,12]. Of central importance is the genetic relatedness of the cooperating individuals: if interactions are with relatives then genes causing altruistic or cooperative behaviour can increase in frequency[5]. While costs of cooperation to individual cells are readily envisaged (expression of traits necessary for cohesion, reduced accessibility of clustered cells to nutrients, build-up of toxic metabolic waste) the selective benefit to forming undifferentiated groups of cells is unclear. Size may be an important factor because larger groups of cells are less prone to predation[2,16,20]; some can migrate further[23]. Recent theory suggests that enhanced resource utilization efficiency and reduced interaction with noncooperative individuals are also relevant[15]. A related issue concerns the existence of spatial structure[13], which increases chances for interactions to occur among genetically related cells[5].

Populations of ancestral smooth (SM) *P. fluorescens* rapidly diversify when propagated in a spatially structured environment (static broth microcosms), generating, via genetic mutation, a range of niche specialist genotypes that are maintained by negative frequency dependent selection[17]. One prominent class of niche specialist is the wrinkly spreader (WS), which colonizes the air–liquid interface. Colonization of this niche enables cells to avoid the anoxic conditions that rapidly build up in unshaken broth culture.

Differences in niche preference of ancestral SM and derived WS genotypes (Fig. 1) led to the hypothesis that WS genotypes owe their

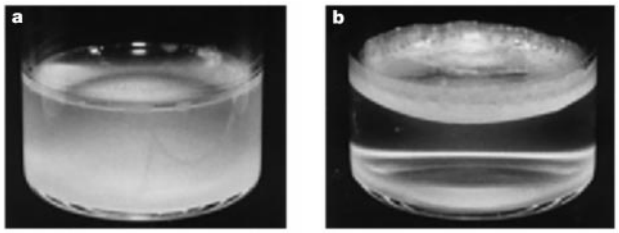

Figure 1 Growth form and niche preference of studied bacteria. **a**, Ancestral (SM) *P. fluorescens* and **b**, derived WS genotypes in static (spatially heterogeneous) microcosms at 25 °C (ref. 17). Only cells of the WS genotype form a mat at the air–broth interface and do so because of a mutation that causes over-production of a polarly expressed polymer. The polymer has glue-like properties and causes daughter cells to remain connected after cell division[18].

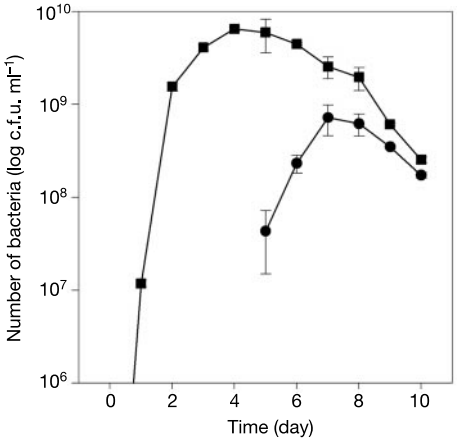

Figure 2 Evolutionary emergence of defecting genotypes from WS during the course of selection in spatially heterogeneous microcosms. The defecting genotypes arise *de novo* by mutation and have a smooth colony morphology that enables them to be readily distinguished from the undulate WS genotypes (see ref. 17). Values are means ± s.e.m of three replicates. Squares represent WS; circles represent defecting genotypes. c.f.u., colony-forming units.

evolutionary success to cooperation (Fig. 1b). If true, then according to theory, selection will favour evolution of the cooperating type provided the costs of cooperation to individual cells are traded against increased fitness at the group level[3–5,11,12].

To measure fitness of individual WS cells, equal numbers of WS and SM cells were competed over 24 h in spatially heterogeneous microcosms. The ratio of malthusian parameters of each competing type was used to calculate the fitness of WS relative to SM[24] During the first 24 h of growth, that is, during the exponential phase (3.5×10^2 to 5.5×10^7 cells ml^{-1}) when resources are abundant, the relative fitness of WS was 0.80 (95% confidence interval, CI: 0.69–0.91, based on the *t* distribution with two degrees of freedom), which is significantly less than 1.0, the fitness by definition of the ancestral genotype.

Despite a much reduced doubling time, WS readily invades (from a single mutant cell) populations dominated by the ancestral genotype to reach population densities that exceed those of the originally dominant ancestral type[17,25]. Its ability to achieve this is attributable to simple mutations that leads to over-production of a cellulosic polymer[18].

Theory predicts that the group will be vulnerable to the evolutionary emergence of defectors, which, in the absence of conflict mediators, stand to weaken the cohesiveness of the group[3,4,11,12]. Vulnerability stems from the fact that selection rewards cells that avoid paying the cost of cooperation—selection especially favours defectors that 'cheat', that is, cells that gain additional advantage from the cooperating type over and above that gained by avoiding the cost of cooperation.

To test this prediction, replicate microcosms were founded with the WS genotype and their evolutionary trajectory followed over a ten-day period (Fig. 2). By day five, mutants that were ancestral-like in terms of colony morphology and niche preference had evolved by mutation; none of these cells showed any tendency to aggregate or form mats and there was no significant difference in competitive fitness between defectors and ancestral SM cells (mean fitness of defectors relative to ancestral SM was 0.96; 95% CI: 0.86–1.05, based on the *t* distribution with two degrees of freedom). Because these mutants no longer act cooperatively we refer to them as defectors.

We next asked whether defectors were cheats. To do this we examined the population dynamics of WS and defector genotypes during the course of a ten-day period of competition. We then compared these to the dynamics of each genotype propagated on its own (Fig. 3). The fitness of defectors was enhanced (at least during the early stages) when propagated in direct competition with WS. This strongly suggests that defectors, while selectively favoured because of their ability to colonize the broth phase[17], enjoy additional rewards that stem from the presence of WS.

Given that defectors arise by mutation from WS, their origins and fate are intimately associated with the group. A defector that has avoided the cost of cooperation, but is nevertheless held within the mat is likely to reap a strong frequency-dependent advantage because it can 'hitchhike' with WS genotypes, reaping the benefits (especially access to oxygen that is otherwise limiting to defectors), while paying none of the costs. Such behaviour on the part of defectors is evident between days 1 and 2, and days 3 and 4 (Fig. 3a).

In apparent contradiction, defectors appear to be at a selective disadvantage between days 2 and 3, but this is attributable to disproportionate death of defector cells in the broth phase caused by anoxia brought about by mat maturation between days 2 and 3. During this time the WS mat changes from a fine film to a robust structure (Fig. 1b) that restricts oxygen diffusion into the broth phase in a manner akin to a layer of oil. This causes death of a significant fraction of the defector cells in the broth. To show this we harvested bacteria from 2- and 3-day microcosms containing competing WS and defector genotypes and determined the number of cells in the broth phase on each occasion. In 2-day microcosms the broth phase contained on average 6.5×10^9 cells ml^{-1} (95% CI: 4.0×10^9 to 9.0×10^9 cells ml^{-1}), but dropped to 1.1×10^8 cells ml^{-1} (95% CI: 2.1×10^8 to 6.5×10^8 cells ml^{-1}) on day 3 (the total number of cells in the mat phase on day 3 being 5×10^9 cells ml^{-1} (95% CI: 2.2×10^9 to 7.8×10^9 cells ml^{-1})). Similar reductions in broth-phase colonizers were obtained by applying a 2-mm layer of oil to 2-day cultures. An alternative explanation for the decrease is the activation of a cryptic phage, but extensive searches revealed no evidence of phage activity.

If cheating, via hitchhiking, goes unchecked, the WS group stands to be undermined[26]. WS genotypes grow unaffected by the presence of cheats through the first four days of selection, even though cheats reap benefit throughout this time (Fig. 3a). In the absence of cheats (Fig. 3b; dotted line) WS gradually declines, primarily due to the success of the mat—it becomes too heavy and gradually sinks into the broth phase where the group suffers the consequences of anoxia. In the presence of cheats, mats collapse abruptly after day 4. This premature collapse is a consequence of the evolutionary success of the cheats and is supported by analyses of the frequency of cheats within the mats: at day 3, 24% of cells within mats are cheats. Because cheats do not contribute to mat integrity their presence is likely to have a negative effect on mat strength. Consistent with this prediction, a cheat-infiltrated mat (24% cheaters) collapses under the weight of 79 mg of glass beads (95% CI: 56–101 mg based on the *t* distribution with two degrees of freedom), whereas a 3-day-old

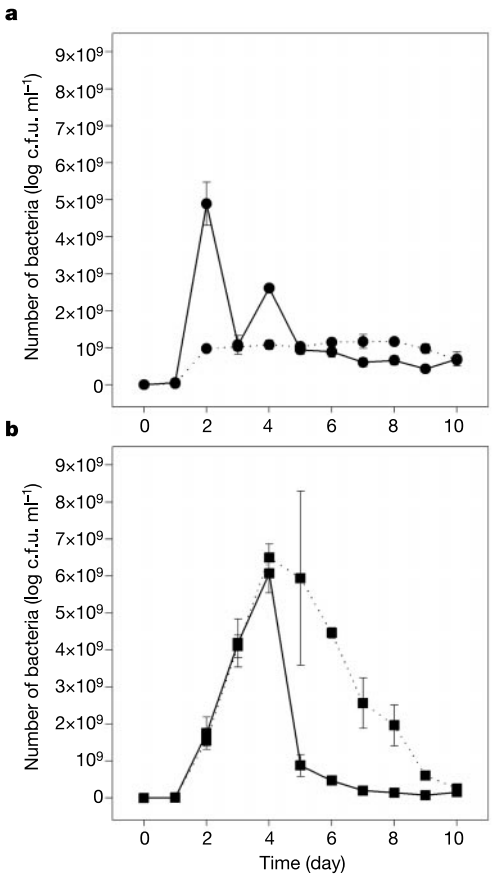

Figure 3 Population dynamics of WS and defector genotypes in the presence and absence of competition. Competing genotypes were founded at equal densities (less than 10^3 cells ml^{-1}). Every 24 h three replicate microcosms were harvested and the frequency of WS and defector genotypes was scored using colony morphology on agar plates to distinguish variant types[17]. The effect of WS on the fitness of defector genotypes (**a**) is the difference between dotted (absence of WS) and solid (presence of competing WS) lines. The effect of defector genotypes on the fitness of WS genotypes (**b**) is the difference between the dotted (absence of competition) and solid (presence of competing defector genotype) lines. Values are means ± s.e.m. ($n = 3$).

mat comprised solely of WS cells supports glass beads to a mass of 432 mg (95% CI: 390–473 mg based on the *t* distribution with two degrees of freedom).

Here we have described an evolutionary transition from individual cells to a cooperating group that occurs *de novo* during the course of selection of *P. fluorescens* in a heterogeneous environment. The transition is dependent upon spatial heterogeneity; competition for resources (primarily oxygen) is the driving force[17,25,27]. The cause of cooperation is a cellulosic polymer that is over-produced by WS cells[18]. Overproduction of the polymer is costly to individual WS cells, but nevertheless the trait spreads by kin selection[5] because causing cells (clones) to adhere to one another promotes colonization of the air–liquid interface. Despite the negative impact of defectors on the evolutionary success of the WS mat, WS are a persistent feature of the evolved populations, emerging afresh after each collapse and maintained by negative frequency-dependent selection[17]. In all respects, our results confirm crucial elements of long-standing theory[1,3–6,8,9,11,12,26].

Undifferentiated groups of WS are a far cry from multicellularity. A likely next step is the evolution of conflict mediators[1,12,28]. The form of these mediators, and the selective conditions necessary for their emergence, is an experimentally tractable problem; and of some significance because the cellulosic polymer both creates the group and has the potential to co-evolve with traits that evolve on the basis of the group[11,29,30].

Finally, the ease and repeatability of this evolutionary transition is notable. Microbiologists have long known that cultures left on the laboratory bench grow a surface scum analogous to a WS mat. Similar selective forces (competition for oxygen) are likely to operate in a range of aqueous environments, possibly resulting in frequent transitions to undifferentiated multicellularity in the wild. Indeed, an accompanying paper supports this conjecture[31] and further highlights the significance of adhesive factors[29]. As such, simple undifferentiated groups of bacteria may have played an important early role in the evolution of multicellularity[2,15], but in addition, cooperative behaviour in bacteria may be more common than currently thought. □

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